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Physiological Response on Broiler Chicken's Liver Supplemented Amino Acid Metionine-Cystine in Feed Contaminated with Aflatoxin B1

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Abstract

The content of AFB1 in feed with low levels and spend a long time, will cause primary damage or primary liver carcinoma. This study aims to reduce the toxicity of AFB1 with amino acid methionine-cystine supplementation in broiler chicken feed. This study used a 3 × 3 factorial design with methionine-cystine amino acid levels (M + C: 75, 100 and 125%) and AFB1 levels (0, 200, and 400 ppb). The variables collected were liver physiology, liver histopathology, SGPT levels, and SGOT levels. Observations of liver physiology showed that feed containing aflatoxin without methionine-cystine amino acid had a paler yellowish color (T4, T7 and T8). Pathological examination resulted that aflatoxicosis will attack the liver. Transition amino acid cystines in chicken feed contaminated with AFB1 did not occur in blood SGPT levels. Blood SGOT levels were highest at 21 days of age, namely T2 (M + C 100%) and T3 (M + C 125%) at AFB1 0 ppb which showed excess liver damage. The administration of methionine-cystine amino acids of 75, 100 and 125% in chicken feed contaminated with AFB1 0, 200 and 400 ppb consumed by broilers carries a risk of physiological and pathological damage to chicken liver.

Keywords: Aflatoxin, liver, SGPT, SGOT

Introduction

Broiler is an industrial commodity that is able to grow quickly and is able to convert feed into meat better than other fowl. An important factor that needs to be considered to success livestock business with increasing livestock productivity is the feed factor. Corn is one of the main commodities in the poultry feed industry. Its use in poultry rations can reach 60% of the total ration. Corn generally cannot be stored for a long time. The condition of Indonesia with a tropical climate with high temperature and humidity will accelerate the decline in the quality of feed ingredients and growth of mold during

Aflatoxin B1 (AFB1) is the mycotoxin of *Aspergillus flavus* fungus which is the most pathogenic and carcinogenic and can affect the health of livestock which causes aflatoxicosis which can inhibit livestock production. In addition, livestock that consume feed contaminated with AFB1 will leave aflatoxin residue in their products which cause negative impact on human health. Low levels of AFB1 content consumed for a long time will cause liver damage or primary liver carcinoma. In addition, aflatoxicosis can also cause vaccination failure, affecting the low production of bile enzymes resulting in decreased fat digestibility and ration efficiency, low

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productivity and anorexia (Lesson and Summers, 2005).

The approach taken in this study was to supplement methionine-cystine amino acids in a ration to reduce the toxic effects that would be caused. Previous research stated that the increase in glutathione content can be done by adding sulfur amino acids, methionine (Yunianta, 2013). Yunianta (2013) reported that the use of methionine amino acid of 0.75 - 1.2% was able to eliminate AFB1 to 1000 ppb and improve production performance The addition of methionine-cysteine amino acids in the ration would be an interesting thing to know their mechanism of binding AFB1 in feed. The aim of this study was to look at the effects of supplementation on physiological responses and the histopathology of broiler chickens's organs that consumed feed contaminated with AFB1.

Materials and Methods

The livestock used in this study were Lohmann MB 202 Platinum day old chicken (DOC) broiler as much as 240 birds which had been vaccinated with Newcastle Desease (ND) live, IB-ND killed and IBD transmune. Day old chicken is obtained from PT. Japfa Comfeed Indonesia. The equipment used in this study was a chicken cage system with a size of 0.5 × 1 m as much as 40 pieces. Cages which has been cleaned, strerilized and desinfected are equipped with a place to feed and drink.

The experimental design used in this study was a 3 × 3 factorial design. The first factor was the administration of methionine-cystine amino acids (75%, 100% and 125%) and the second factor was aflatoxin levels (0, 200, and 400 ppb) in the chicken ration. Each treatment consists of 4 or 5 replications. The levels of methionine-cystine given were 75% (0.71), 100% (0.92) and 125% (1,175) (Aviagen, 2014). The feed treatments given are:

T1 = ration (M+C) 75% + AFB1 0 ppb
T2 = ration (M+C) 100% (normal) + AFB1 0 ppb
T3 = ration (M+C) 125% + AFB10 ppb
T4 = ration (M+C) 75% + AFB1200 ppb
T5 = ration (M+C) 100% (normal) + AFB1200 ppb
T6 = ration (M+C) 125% + AFB1200 ppb
T7 = ration (M+C) 75% + AFB1400 ppb
T8 = ration (M+C) 100% (normal) + AFB1400 ppb
T9 = ration (M+C) 125% + AFB1400 ppb

Note: M : Methionine, C: Cystine, AFB : Aflatoxin B

A total of 240 birds were grouped in nine treatments and housed in Poultry Science Laboratory, Faculty of Animal Husbandry, Universitas Gadjah Mada. Treatment starts from DOC (0-21 days).

Chicken samples were taken at the age of 14 and 21 days. Each treatment was taken three chickens from different cages. Blood samples were taken from the blood that came out during the slaughter process and were inserted into a purple vacutainer tube that contained EDTA to obtain blood plasma. The liver was stored in 10% formalin and then tested for liver necrosis by making histological preparations in the Anatomy Laboratory, Faculty of Medicine, Universitas Brawijaya. Blood samples were tested by SGPT and SGOT and liver samples were carried out liver histopathology test to see whether there was liver necrosis. The total sample taken was 27 samples. The changes measured in this study were liver physiology, liver histopathology, SGPT and SGOT levels.

Data is processed by analysis of various analysis of variance (ANOVA) using SPSS software ver. 15. If the treatment has a significant effect on the observed variables, then proceed with Duncan's multiple range test (DMRT) (Mattjik and Sumertajaya, 2002). Liver physiology and histopathology data are discussed descriptively. SGPT and SGOT tests were carried out by taking a 5 ml blood sample in vacutest and then centrifuging it to get the plasm. Blood plasma was measured by SGPT and SGOT using a spectrophotometer, serum levels expressed in UI / L.

Result and Discussion

Results of internal organ observation

The results of chicken's internal organs observation in this study showed that the physiology of chicken liver found aflatoxicosis in high AFB1 treatment and low M + C. The results of statistical tests of factorial patterns on the relative weights of the liver did not show significantly different as the effects of giving M + C, AFB1, or the interaction between the two in all age groups. Ahmad (2009) explained that aflatoxin causes a variety of organ weights, namely enlargement of the liver, spleen, kidney, fatty liver syndrome, reduction of bursa fabricius and thymus, changes in the texture and color of the liver, hemorrhage, immunosuppression, and necrosis.

Liver

One symptom of aflatoxicosis can be seen in internal organs such as liver. Liver is the main place for detoxification of poisons that enter the body. Chickens that consume feed containing AFB1 cause the liver to physiologically change color. The high content of aflatoxin shows a paler yellowish color. Liver images on each treatment aged 14 day old are presented in Figure 1 and Figure 2 aged 21 days old chickens. Observations of liver physiology were carried out descriptively by looking at the color differences.

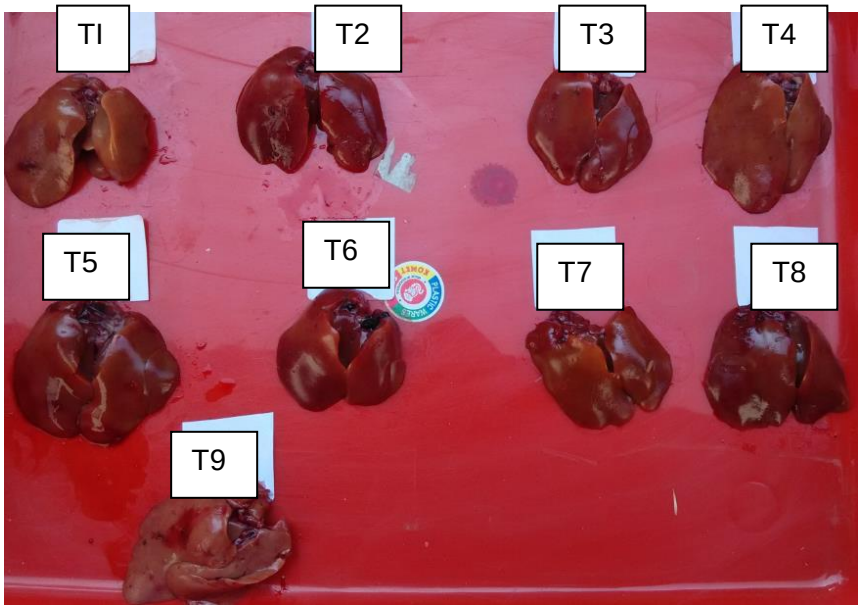

Figure 1. Physiology of liver on each treatment aged 14 days old.

Figure 1 shows that chicken livers with low M + C content and high AFB1 have a pale yellowish color as seen in T1, T4, and T7. Exposure to AFB1 at the age of 14 days has begun to show symptoms of aflatoxicosis even though the color is not pale yellow as shown in Figure 2 where AFB1 exposure lasts longer, which is 21 days. Aflatoxicosis in chickens causes the liver to be yellow with multi-focal hemorrhage and uneven surface on the liver capsule (Smela *et al*., 2001). Color differences can occur due to gradual accumulation of fat (infiltration), of fat in hepatocyte cells (Valdivia et al., 2000). Fatty liver generally appears from the color of the liver to yellowish. Aflatoxin B1 is hepatotoxic with fatty changes, hepatocyte degeneration, necrosis and damage to liver function. Liver became pale, swollen and the texture changes. This syndrome in chickens is called fatty liver syndrome (Devegowda and Murthy, 2005).

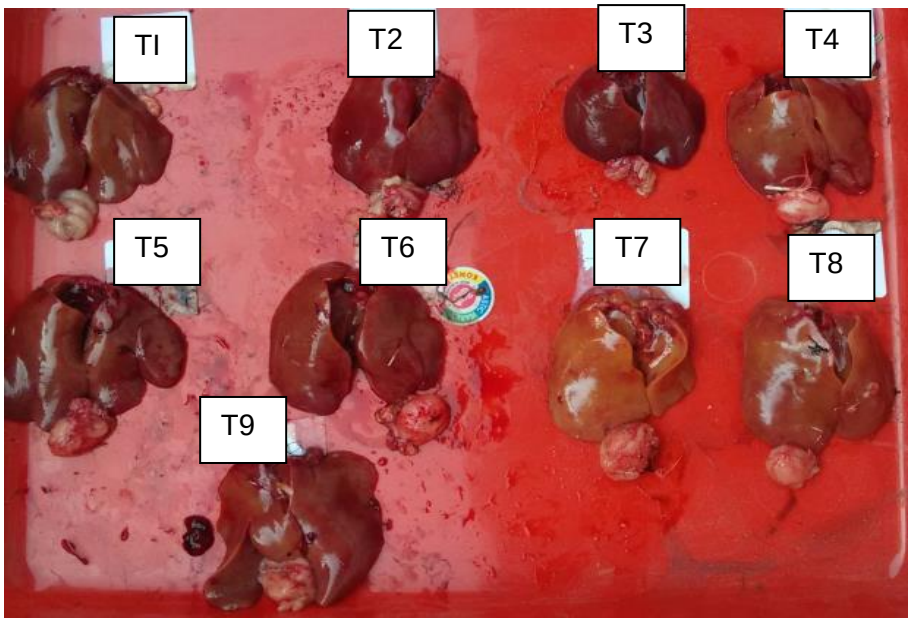

Figure 2. Physiology of liver on each treatment aged 21 days old.

Color changes due to AFB1 can be prevented by adding M + C in the feed. Giving M + C to the feed can improve the color of the chicken liver to normal as shown in Figure 2. In treatment T9 (400 ppb aflatoxin and M + C 125%) the liver color is darker than treatment T7 (400 ppb aflatoxin and M + C 75%). The T7 treatment shows a pale yellow liver. Yellow color is caused by the presence of fat into the liver parenchymal cells (Patriana and Pribadi, 1996; Smela *et al*., 2001) caused by oxidative stress, resulting in an increase in lipid peroxidation and fatty liver which causes yellowing and weight gain (Susanto, 2014). Giving M + C is able to conjugate aflatoxin B1 so that it does not cause liver damage which begins with pale color. The mechanism for eliminating aflatoxin with the addition of M + C can be seen in figure 3. Methionine and cystine are sulfur amino acids which are also precursors of gluthation which play an important role in the conjugation of AFB1-epoxidase. Cysteine is a distal intermediate precursor of glutathione and methionine (Nahm, 1995). Glutathione plays an important role in the AFB1 epoxide conjugation. The conjunctions require the starting material, namely the amino acid methionine and cysteine which produces N-acetyl cysteine which can increase the level of glutathione S-transferase (GST).

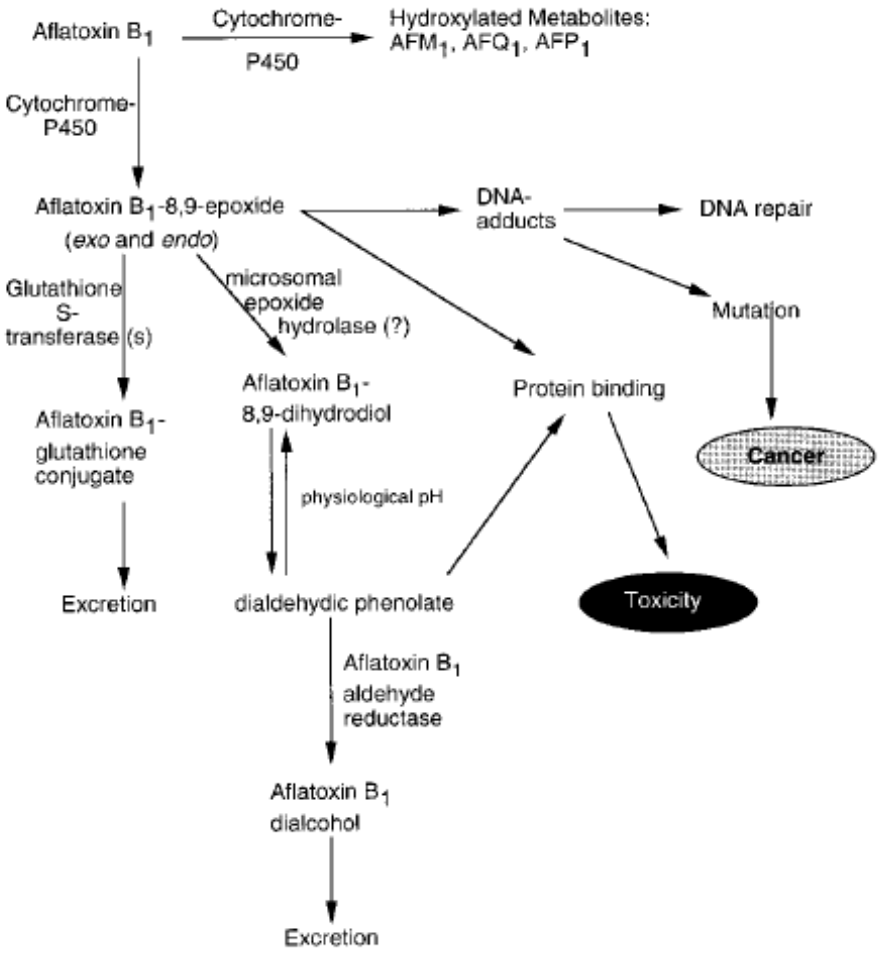

Figure 3. Scheme of function of biotransformation process and bioactivation of AFB1 (Bammler *et al.,* 2000).

Aflatoxin B1 has high acceleration by forming epoxides and the speed of conjugation is low with glutathione (Pestka and Smolinski, 2005). The glutathione conjugation is the binding of electrophile carbon to the substrate by the sulfihidril group present in the glutathione group. Intermediate compounds that bind to glutathione will prevent intermediate compounds with liver macromolecules, so that liver cell damage can be avoided. Liver damage decreased resulting in the liver which still work optimally to secrete enzymes that are useful in the metabolic process (Widiastuti, 2014).

In addition to physiological changes, liver damage can also be seen from liver histology by staining hematoxylin eosin (HE) in the cross section of the liver of broiler chicken. Histopathological observations on HE staining preparations were carried out descriptively by seeing whether there were changes in the tissue under the

microscope. The effect of AFB1 on animals can cause pathological changes in the liver. The liver becomes yellowish and blotchy, cell degeneration, necrosis and proliferation of the beliferus duct (Yunus et al., 2011). Figures 5 to 13 are histologies of 21-day-old chicken liver with 100x magnification.

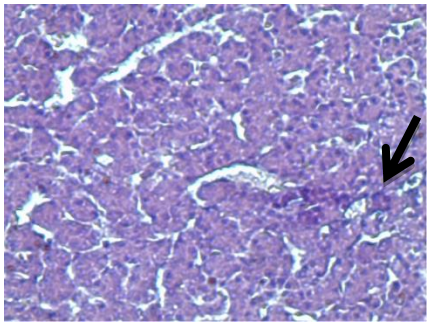

Figure 5. Treatment T1 (inflamed)

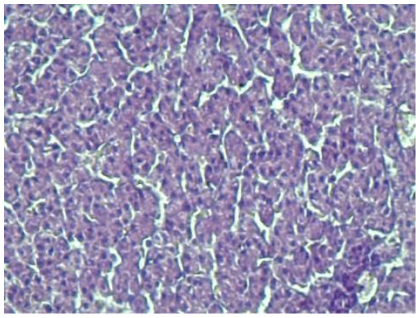

Figure 6. Treatment T2 (normal)

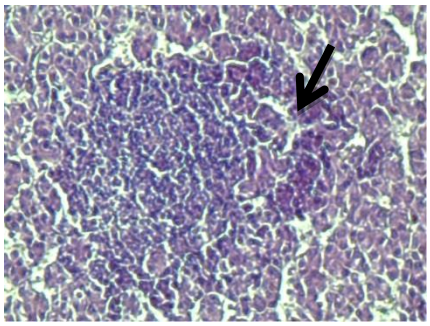

Figure 7. Treatment T3 (inflamed)

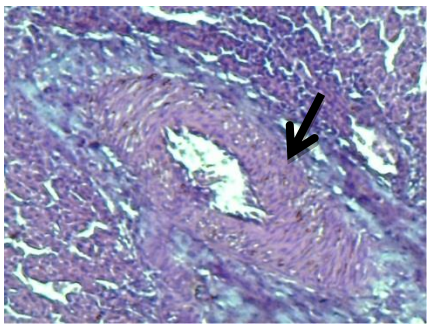

Figure 8. Treatment T4 (dam like)

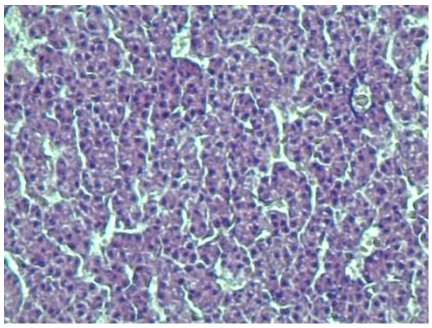

Figure 9. Treatment T5 (normal)

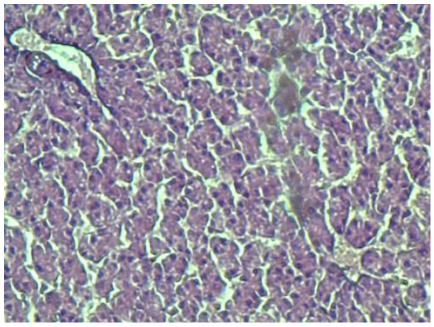

Figure 10. Treatment T6 (normal)

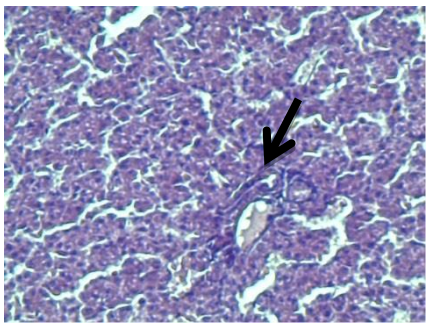

Figure 11. Treatment T7 (lipid in liver)

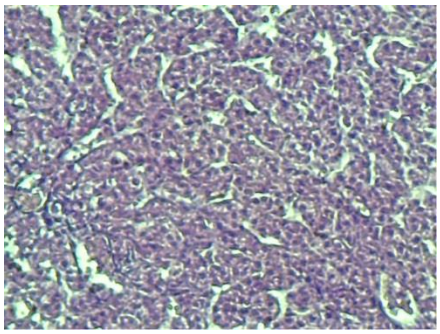

Figure 12. Treatment T8 (normal)

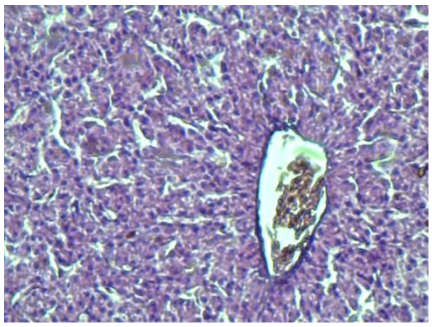

Figure 13. Treatment T9 (normal)

The results of the analysis were carried out at the Anatomy Laboratory, Faculty of Medicine, Universitas Brawijaya by the method carried out as in the research of Sugito et al. (2006). Liver histopathology results show that the liver is normal in treatments T2, T5, T6, T8 and T9. The appearance of inflammation occurs in the treatment of T1 and T3 which causes the liver to be unable to work optimally so that body weight gain in treatments T1 (M + C 75% and AFB1 0 ppb) and T3 (M + C 125% and AFB1 0 ppb) are lowest. Fatty liver occurs in the T7 treatment which causes the liver to be more yellow. In this treatment M + C is given at the lowest level, namely 75% and AFB1 is given at the highest 400 ppb. In treatment T4 with level M + C 75% and AFB1 200 ppb occured damming.

Pathological examination results that aflatoxicosis will attack the liver. Aflatoxicosis in livestock will experience hemorrhage, hepatic necrosis and bile vessel proliferation. Liver cells trapped by the proliferation of bile vessels and connective tissue experience fat degeneration (Priosoeryanto et al., 2002) as seen in treatment T7. Six chickens liver samples from each treatment that were tested showed that not all parts were damaged, so the liver could still work well. This is also supported by the SGPT results in Table 1 which tell about enzyme that can detect the presence of liver damage in this study; and resulted no significantly different at the age of 14 or 21 days.

***Serum Glutamine Pyruvate Transaminase* (SGPT) and *Serum Glutamine Oxaloacetat Transaminase* (SGOT)**

Serum Glutamine Pyruvate Transaminase (SGPT) is an enzyme in the liver that functions to carry out enzymatic reactions to neutralize toxins that enter the liver which are secreted in blood serum. Blood test results of broiler chicken aged 14 and 21 can be seen in Table 1. The results of factorial pattern analysis showed that the interaction effect between M + C and AFB1 on SGPT in chicken blood in this study was not detected, both at 14 and 21 days.

Table 1. Blood SGPT test of broiler chicken aged 14 and 21 days (µ/L)

AFB1	M+C			
	75%	100%	125%	Average
Aged 14 days				
0 ppb	3,83 ± 0,35	4,93 ± 1,79	5,23 ± 1,29	4,67 ± 1,29
200 ppb	4,37 ± 0,61	5,33 ± 3,44	8,37 ± 2,71	6,02 ± 2,86
400 ppb	4,40 ± 1,25	5,17 ± 1,32	5,07 ± 1,10	4,88 ± 1,12
Average	4,20 ± 0,77	5,14 ± 2,06	6,22 ± 2,27	
Aged 21 days				
0 ppb	4,87 ± 1,75	4,87 ± 1,68	3,77 ± 0,59	4,50 ± 1,36
200 ppb	3,60 ± 0,70	4,50 ± 0,70	3,33 ± 0,35	3,81 ± 0,75
400 ppb	3,20 ± 0,56	3,57 ± 1,12	5,30 ± 0,14	3,86 ± 1,12
Average	3,89 ± 1,24	4,31 ± 1,21	3,99 ± 0,91	

Chicken blood that has been taken from the slaughter process is put into a tube that already contains EDTA then stored in the refrigerator until it is ready for testing at the LPPT UGM. Aflatoxicosis can be seen from damage of liver cells. SGPT levels showed differences in chicken blood due to the treatment of levels of M + C and AFB1 and did not show any interaction between the two treatments. SGPT levels are normal in this study because they are below 10 µ / L (Utami, 2009). AFB1 levels given are still relatively low, causing the body to be able to detoxify and the liver does not show any damage. In contrast to Utami's research (2009) where SGPT levels were increased in treated chickens AFB1 1000 ppb (14.38 µ / L) and AFB1 1500 ppb (14.83 µ / L). This difference is caused by the ability of chickens to adapt the administration of AFB1 levels (200 and 400 ppb) on their feed.

Serum Glutamine Pyruvate Transaminases are specific in monitoring liver damage due to inflammation or necrosis. The results of the study did not show any liver damage. Damage of liver cells will be characterized by the release of liver enzymes so that the levels of liver enzymes will increase in the blood plasma (Karakilcik et al., 2004). The aflatoxin detoxification process will cause SGPT levels and Serum Glutamine Oxaloacetate Transaminase (SGOT) increase. When liver damage or liver disease occur, SGPT increases over SGOT (Maryam, 1996; Talwar and Srivastava, 2004) gives symptom like liver necrosis (Transito et al., 2011).

Beside SGPT test, SGOT test was performed as they have the same the same function, which is to know liver damage by looking at the SGOT content in the blood. SGOT result of chickens aged 14 and 21 days can be seen in Table 1. The results of the factorial

pattern analysis showed that SGOT values increased (P <0.05) with increasing levels of M + C given in feed at the age of 21 days. SGOT levels also increased (P <0.05) as AFB1 being given at lower levels in both at age 14 and 21 days. The interaction effect between M + C and AFB1 on SGOT chicken in this study was significant (P <0.05) at the age of 21 days.

Table 2 shows that the more of M + C administration from 75 to 125% at the age of 21 days, the more SGOT value of chicken blood. The high value of SGOT at the age of 21 days indicates liver damage. The damage happened due to the high content of M + C which causes the accumulation of methionine changes to homocysteine. The high level of homocysteine with no prevention of diseases or infections such as aflatoxin causes homocysteine to be stored only. Deposits of homocysteine cause another disease called homocysteinemia (heart failure) so that chickens in T2 and T3 treatments have a high mortality rate of 12.5 and 10%.

Table 1. Blood SGOT test of broiler chicken aged 14 and 21 days (µ/L)

AFB1	M+C			
	75%	100%	125%	Average
Aged 14 days				
0 ppb	181,2 ± 41,2	258,3 ± 43,0	258,9 ± 43,0	232,8 ± 53,3^{l}
200 ppb	265,5 ± 59,0	273,5 ± 92,7	285,4 ± 51,4	274,8 ± 61,2^{k}
400 ppb	218,1 ± 26,4	222,7 ± 18,5	194,9 ± 31,6	211,9 ± 25,9^{l}
Average	221,9 ± 53,0	251,5 ± 56,6	246,4 ± 54,7	
Aged 21 days*				
0 ppb	220,7 ± 56,4^{yz}	344,3 ± 24,4^{x}	333,0 ± 29,4^{x}	299,3± 68,3^{k}
200 ppb	179,4 ± 27,8^{z}	213,4 ± 54,2^{yz}	281,3 ± 14,4^{xy}	224,7± 54,7^{l}
400 ppb	176,4 ± 11,5^{z}	228,8 ± 12,1^{yz}	189,9 ± 13,6^{yz}	199,4± 26,9^{l}
Average	192,2 ± 38,5^{b}	262,2± 68,9^{b}	277,8 ± 62,0^{a}	

^{a,b,c} Different superscript in the same row and age, show a significant difference (P<0,05) on M+C level
^{k,l,m} Different superscript in the same row and age, show a significant difference (P<0,05) on AFB1 level
^{x,y,z} Different superscript in the same row and age, show a significant interaction (P<0,05)between M+C level and AFB1 level

SGOT value increases at the lower level of AFB1 in feed, both at the age of 14 and 21 days. SGOT levels at the age of 14 days were highest in AFB1 200 ppb and 0 ppb at 21 days. This is occured due to accumulation of M + C from the feed which cause liver failure. The accumulation of M + C that causes hyperhomocysteinemia is obvious from interactions at the age of 21 days.

The interaction of M + C and AFB1 at the highest SGOT levels was seen in the treatment of T2 (M + C 100% and AFB1 0 ppb) and T3 (M + C 125% and AFB1 0 ppb) which showed liver damage. A higher SGOT value is an

indication of poor liver health, because this enzyme will be produced when the liver is damaged (Nugrahani, 2013). It happened because the feed contains low AFB1 and high M + C causes a buildup of homocysteine which causes hyperhomocysteinemia or known as liver attack. SGOT increase due to tissue damage such as mycocardial infraction, muscle necrosis, kidney disorders, cerebral disorders, and intravascular hemolysis. In this disease, SGOT concentration is higher than SGPT. But in liver disease, SGPT increases beyond SGOT (Talwar and Srivastava, 2004). Since tissue damage is suspected as mycocardial infraction, in the same treatment shows SGPT content is normal while SGOT is high.

Conclusion

From the results of this study it can be concluded that the combination of methionine-cystine amino acids of 75, 100 and 125% and AFB1 0, 200 and 400 ppb consumed by broilers, has not been able to improve the performance of internal organs, namely the liver. Liver physiology showed that the color of chicken's liver fed high aflatoxin (400 ppb) and the low amino acid methionine-cystine (75%) was more pale yellowish. It showed an initial symptom of aflatoxicosis namely fat degeneration. The content of AFB1 in feed with low levels and consumed for a long time, will cause liver damage or primary liver carcinoma.

References

Ahmad, R. Z. 2009. Cemaran kapang pada pakan dan pangendaliannya. J. Litbang Pertanian. 28 (1) : 15 – 22.

Bammler, T. K., D.H. Slone, and D.L Eaton. 2000. Effects of dietary oltipraz and ethoxyquin on Aflatoxin B1 biotransformation in Non-Human Primates. J. Toxicol. Sci. 54. 30 – 41.

Devegowda, G. and T. N. K. Murthy. 2005. Mycotoxins : Their Effect in Poultry and Some Practical Solution. In The Mycotoxin Blue Book. Edited by D. E. Diaz. Nottingham, University Press : 25 – 55.

Karakilcik, A. Z., M. Zerin, O. Arslan, Y. Nazligul, and H. Vural. 2004. Effects of vitamin C and E on liver enzymes and biochemical parameters of rabbits exposed to aflatoxin B1. Vet Human Toxicol46(4): 190 – 192.

Maryam, R. 1996. Residu aflatoksin dan metabolitnya dalam daging dan hati ayam. Prosiding Temu Ilmiah nasional Bidang Veteriner. Bogor, 336 – 339.

Mattjik, A. A. dan Sumertajaya. 2002. Perancangan Percobaan Dengan Aplikasi SAS dan Minitab Jilid 1 Edisi Kedua. IPB Press, Bogor.

Nahm K. H. 1995. Possibilites for preventing mycotoxicosis in domistic fowl. J. Word's Poultry Sci. 51: 177 – 185.

Nugrahani, W. 2013. Konsumsi minuman beroksigen dan dampaknya terhadap performa saat berolahraga, profil lipid, glukosa darah dan SGOT/SGPT. Tesis. Program Studi Ilmu Pangan

Fakultas Teknik Pertanian. Institut Pertanian Bogor.

Patriana, U. dan E. S. Pribadi. 1996. Studi mengenai sifat akumulasi aflatoksin pada organ jeroan ayam yang mendapatkan suntikan kortikosteroid dan antibiotik. Med. Vet. 3(1): 43 – 52.

Pestka, J. J. and A. T. Smolinski. 2005. Deoxynivalenol: Toxicology and potential effect on humans. J. Toxicol. Environ. Health Brit. Rev. 8 : 39 – 69.

Priosoeryanto, B. P., H. Huminto, S. Estuningsih, E. Harlina, dan R. Tiuria. 2002. Aflatoksikosis pada itik ras pedaging dan ras lokal di suatu peternakan di Cianjur, Jawa Barat. J Mikol. Kedok. Ind.3(1-2): 24 – 27.

Smela, M. E., S. S. Curier, E. A. Bailey, and J. M. Essingmann. 2001. The Chemistry and biology of aflatoksin B1. Carcinogenesis 22 (4):535 – 545.

Sugito, W. Manulu, D. A. Astuti, E. Handharyani, dan Chairul. 2006. Histopatologi hati dan ginjal pada ayam broiler yang dipapar cekaman panas dan diberi ekstrak kulit batang jaloh (*Salix tetrasperma roxb*). Semnas. Teknol. Peternakan dan Vet: 728 – 734.

Susanto, A. 2014. Glukomanan dari iles-iles (*Amorphopalus ancophylus*) sebagai pengikat aflatoksin pada pakan ayam broiler. Disertasi. Fakultas Peternakan. Institut Pertanian Bogor, Bogor.

Talwar, G. P. and L. M. Srivastava. 2004. Textbook of Biochemistry And Human Biology. 3rd Ed. Prentice-Hall Pvt.Ltd, India.

Transito, L. S., J. C. D. Rio-Garcia, J. L. Arjona-Roman, E. Moreno-Martinez, and A. Mendez-Albores. 2011. Effect of citric acid supplemented diets on aflatoxin degradation growth performance and serum parameters in broiler chickens. Arch. Med. Vet. 43: 215 – 222.

Utami, M. M. D., 2009. Efektivitas Ekstrak Bawang Putih dalam Pakan untuk Detoksifikasi Aflatoksin B1 pada Ayam Broiler. Tesis. Fakultas Peternakan. Universitas Gadjah Mada. Yogyakarta.

Valvida, A. G., A. Martinez, F. J. Damian, T. Quezda, R. Ortiz, C. Martinez, J. Lamas, L. Rodriguez, L. Yamamoto, F. Jaramillo, M.G. Loarca-Pina, and J. L. Reyes. 2001. Efficacy of N-Acetylcysteine to reduce the effects of Aflatoxin B1 Intoxication in Broiler Chicks. J. Poultry Sci. 80: 727 – 734.

Widiastuti, R. 2014. Residu aflatoksin dan metabolitnya pada berbagai produk pangan asal hewan dan pencegahannya. Wartazoa 24 (3): 179 – 190.

Yunianta. 2013. Upaya Penurunan Tingkat Toksisitas Aflatoksin B1 pada Jagung serta Penggunaannya Sebagai Pakan Broiler. Tesis. Fakultas Peternakan. Universitas Gadjah Mada, Yogyakarta.

Yunus, A. W., E. Razzazi-Fazeli, and J. Bohm. 2011. Aflatoxin B1 in affecting Broiler's performance, immunity, and gastrointestinal tract: A review of history and contemporary issues. J. Toxins 3: 566 – 590.